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REGENERATION FROM LONG-TERM EMBRYOGENIC CALLUS OF THE *ROSA HYBRIDA* CULTIVAR KARDINAL

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Summary

Media components used for three stages of development: (1) callus maintenance, (2) maturation of embryos, and (3) conversion of embryos to plants were shown to affect regeneration of plants for the commercially important red rose cultivar Kardinal. Embryogenic callus was maintained for 5 yr on either Schenk and Hildebrandt's basal salts medium (SH) supplemented with 13.6 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) or Murashige and Skoog's basal salts medium (MS) supplemented with 18.1 μ*M* dicamba and 0.46 μ*M* kinetin. Maturation of embryos was three times higher using callus maintained on the SH medium supplemented with 2,4-D while conversion of cotyledonary-stage embryos to plants was significantly higher (10 times) using callus that had been maintained on MS medium with dicamba and kinetin. Maximum maturation (13.5%) and conversion (15.2%) occurred when callus was cultured on MS maturation medium without hormones. Cotyledonary-stage embryos cultured on MS conversion medium supplemented with abscisic acid (5–20 μ*M*) produced plants that survived at a significantly higher rate (two times) in the greenhouse than when embryos were cultured without abscisic acid. The highest rate of plant regeneration occurred when embryogenic callus of 'Kardinal' was maintained on MS medium supplemented with dicamba and kinetin, maturation of embryos occurred on MS maturation medium without hormones, and conversion of cotyledonary-stage embryos occurred on MS conversion medium supplemented with abscisic acid.

Key words: abscisic acid; conversion; hybrid tea roses; regeneration.

Introduction

Regeneration of roses from embryogenic callus has been reported for hybrid teas (De Wit et al., 1990; Noriega and Sondahl, 1991; Firoozabady et al., 1994; Hsia and Korban, 1996; Murali et al., 1996; Kintzios et al., 1999; Sarasan et al., 2001; Castillon and Kamo, 2002; Li et al., 2002a), floribunda and other garden-type roses (Kunitake et al., 1993; Marchant et al., 1996; Castillon and Kamo, 2002), and rootstock roses (van der Salm et al., 1996; Castillon and Kamo, 2002). Each publication describes a different cultivar, and the *in vitro* conditions used for regeneration also differ as reviewed (Rout et al., 1999). A few studies have determined the factors that affect maturation and conversion of the somatic embryos, but it is difficult to comprehend the importance of specific factors in the regeneration process.

This study focused on the commercially important red hybrid tea cultivar Kardinal because this cultivar should be useful in future transformation studies due to its commercial importance and relatively high rate of regeneration. Our study used embryogenic callus that had been maintained for 4–5 yr on either MS medium (Murashige and Skoog, 1962) supplemented with 18.1 μ*M* dicamba and 0.46 μ*M* kinetin (MS DCK) or SH medium (Schenk and Hildebrandt, 1972) supplemented with 13.6 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) (SH 2,4-D). Others (Derks et al., 1995; Marchant et al., 1996; van der Salm et al., 1996) have used SH medium with up to 50 μ*M* 2,4-D for induction and maintenance of embryogenic callus. Marchant et al. (1996) showed that the frequency of embryogenic callus induction from both petiole and root explants was higher on SH medium, rather than MS medium, with 2,4-D. They recommended SH medium supplemented with 2,4-D for maintenance of embryogenic callus. We used MS medium supplemented with dicamba and kinetin, as long-term exposure to 2,4-D can inhibit plant regeneration and result in phenotypically abnormal plants.

Successful transformation of rose has been achieved by others using either primary callus (Li et al., 2002b), callus that had been cryopreserved (Firoozabady et al., 1994), or stem explants that were then induced to form callus (van der Salm et al., 1997) as these methods will avoid the decrease in regeneration frequency that typically occurs with time in culture. This is the first study on regeneration from long-term (4–5-yr-old) callus cultures of rose, and it shows that 'Kardinal' callus maintains its regeneration capacity for a long period of time. The medium used to maintain the callus cultures for 5 yr was found to affect regeneration of 'Kardinal.'

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Abscisic acid (ABA) has been shown to benefit somatic embryo development of both woody and herbaceous species, particularly woody species (Merkle et al., 1995). Somatic embryos of several species of Norway, white, and interior spruce that matured on ABA showed levels and accumulation of storage proteins similar to that of zygotic embryos, as compared to somatic embryos that had not matured on ABA (Misra et al., 1993; Dunstan et al., 1998). Other effects of ABA include triglyceride accumulation in somatic embryos of spruce and stimulation of quiescence for somatic embryos of alfalfa (Merkle et al., 1995).

ABA did not positively affect maturation or conversion of 'Kardinal' somatic embryos, although ABA was reported as necessary for conversion of the hybrid teas 'Arizona' and 'Carefree Beauty' (Murali et al., 1996; Li et al., 2002a). Marchant et al. (1996) included ABA in their medium for maturation of embryos but did not provide data showing the necessity of ABA. In this study the role of ABA on maturation and conversion of somatic embryos and overall growth of regenerated 'Kardinal' plants was examined.

Materials and Methods

Embryogenic callus cultures. Embryogenic callus from the cultivar Kardinal was induced from root explants according to Derks et al. (1995). Callus was maintained for 5 yr on either MS DCK medium [Murashige and Skoog's basal salts (M-5524, Sigma Chemical, St. Louis, MO) supplemented with 3% (w/v) sucrose, 18.1 μ*M* 3,6-dichloro-*o*-anisic acid (dicamba), 0.46 μ*M* kinetin, 26.7 μ*M* glycine, 0.29 μ*M* thiamine, 4.06 μ*M* nicotinic acid, 2.4 μ*M* pyridoxine, 555.6 μ*M* *myo*-inositol, and solidified with 0.25% (w/v) Phytagel (Sigma Chemical Co.)] or SH 2,4-D medium [Schenk and Hildebrandt's basal salts (S-3766, Sigma Chemical Co.) supplemented with 3% (w/v) sucrose, 13.6 μ*M* 2,4-D, 0.3 μ*M* thiamine, 4.1 μ*M* nicotinic acid, 2.4 μ*M* pyridoxine, and 2.6 m*M* L-proline]. All media were adjusted to pH 5.8 prior to autoclaving at 120 kPa, 121 °C for 20 min. Callus cultures were transferred monthly and maintained in the dark at 25 °C.

Embryo maturation. Embryogenic callus that had been maintained on MS DCK medium was dispersed and filtered prior to culture on MS maturation medium for all experiments unless stated otherwise. MS maturation medium consists of MS basal salts supplemented with 0.29 μ*M* thiamine, 4.06 μ*M* nicotinic acid, 2.4 μ*M* pyridoxine, 3% sucrose, 555.6 μ*M* *myo*-inositol, 26.7 μ*M* glycine, and solidified with 0.25% Phytagel, pH 5.8.

Approximately 20 g fresh weight (FW) of embryogenic callus was placed in a baffled 250 ml flask with 50 ml of liquid MS medium without hormones, then shaken on an orbital shaker at 120 rpm for 3 h in the dark at 25°C to disperse the callus cells. The suspension was filtered sequentially through stainless steel screens of mesh sizes 2000, 1180, and 850 μm, using 100 ml of liquid MS medium lacking hormones to wash cells through each screen. Cells that passed through the 850 μm screen were resuspended in 100 ml of liquid MS medium lacking hormones and then 2 ml aliquots were collected on a Whatman no. 1 filter paper of 7 cm diameter. Liquid was removed from the filter paper by placing the filter paper on a Buchner funnel and then applying a gentle vacuum. The filter paper and its cells were placed on maturation medium in Petri plates (100 × 20 mm). A FW measurement was taken for three filter papers each containing a 2 ml aliquot of the cells following application of a vacuum to remove excess liquid from the filter paper and cells. The average fresh weight for the 2 ml aliquot of cells was used as the basis for calculating the number of cotyledonary-stage embryos that developed from each plate.

The filtered and dispersed cells were used for two experiments. In the first experiment comparing callus that had been maintained on either MS DCK or SH 2,4-D medium, the filtered callus was cultured on MS maturation medium.

The second experiment compared MS maturation media with or without 0.25% activated charcoal (AC) and SH maturation medium (same as MS maturation medium except with SH basal salts) with AC.

Cultures were grown on one of the three maturation media in the dark at 25°C for 8 wk, at which time the number of cotyledonary-stage embryos was counted using a dissecting microscope. Only embryos with cotyledons at least 2 mm in diameter were counted as mature, cotyledonary-stage embryos. Maturation of cotyledonary-stage embryos continued for an additional 2–3 wk on the original maturation medium without subculturing, and these embryos were used for conversion experiments.

Conversion of cotyledonary-stage embryos. Cotyledonary-stage embryos were transferred to MS conversion medium [same as MS maturation medium except supplemented with 86 μ*M* ethylenediamine-di(o-hydroxyphenyl-acetic acid) (FeEDDHA) (van der Salm et al., 1994)] where they were placed under cool-white fluorescent lights (40–60 $\mu mol\, m^{-2}\, s^{-1}$) with a 12 h light photoperiod. Each Magenta jar contained 50 ml of medium. Filter-sterilized ABA (Sigma Chemical Co.) was added cold-sterilely to MS conversion medium at a final concentration of either 5, 10, or 20 μ*M* ABA. The percentage of embryos that converted was determined by counting the number of embryos that developed both shoots and roots after 6 wk on MS conversion medium. Plants grown for 6 wk on MS conversion medium were dried overnight at 60°C for a dry weight (DW) measurement. Ten plants grown on each ABA concentration were weighed. Plants were grown for a further 2 mo. before being grown in the greenhouse.

Growth in the greenhouse. In May, plants were transferred from Magenta jars to pots containing Metro Mix 510 (Scotts Company, Marysville, OH) and placed under a mist bench in the greenhouse for approximately 1–2 mo. When new growth of the shoot was well established, the plants were removed from the mist bench. Two months later the plants that survived were counted, the number of plants at the flowering stage, heights of the plants, and leaf measurements were taken. The width and length of 20 leaves, each from a single plant, that were grown on each ABA concentration were measured. The greenhouse was maintained at 24–26 °C/21–23 °C (day/night).

Statistical analysis. Maturation of somatic embryos (Tables 1 and 2) was determined using two filtrations performed separately. Each replication consisted of 20 plates, and each Petri plate contained a 2 ml aliquot of cells. All embryos that matured to cotyledonary-stage embryos were cultured on conversion medium, and the number of cotyledonary-stage embryos that converted to intact plants with at least one root was determined.

TABLE 1

MATURATION AND CONVERSION FROM SOMATIC EMBRYOS OF *ROSA HYBRIDA* 'KARDINAL' DERIVED FROM EMBRYOGENIC CALLUS MAINTAINED ON EITHER MS DCK OR SH 2,4-D MEDIA

Maintenance media	Embryo maturation[y]	Conversion to plants[z]
MS DCK	2.8 a	30.0 a
SH 2,4-D	8.1 b	3.1 b

[z] Percent cotyledonary-stage embryos.

[y] Number of cotyledonary-stage embryos per 10 mg FW callus.

Means within each *column* that are shown with different *letters* are significant at either $P < 0.01$ for embryo maturation or $P \leq 0.05$ for conversion to plants, according to the *t*-test (unequal variance).

TABLE 2

MATURATION AND CONVERSION OF 'KARDINAL' EMBRYOS CULTURED ON EITHER SH OR MS MATURATION MEDIA WITH AC OR MS MATURATION MEDIUM WITHOUT AC

Maturation media	Embryo maturation[y]	Conversion to plants[z]
MS − AC	13.5 a	11.0 a
MS + AC	1.4 b	15.2 a
SH + AC	1.6 b	2.9 b

[z] Percent cotyledonary-stage embryos.

[y] Number of cotyledonary-stage embryos per 10 mg FW callus.

One-way analysis of variance showed $P \leq 0.001$ for embryo maturation and $P = 0.003$ for conversion. Means within each *column* that are shown with different *letters* are significant at $P \leq 0.05$ according to Dunn's multiple comparison.

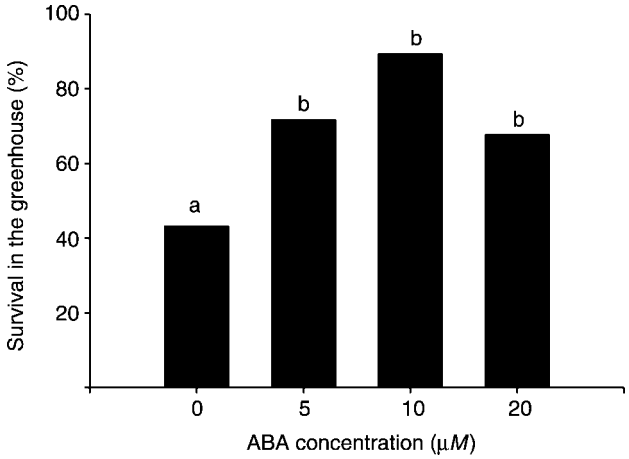

FIG. 1. Survival of embryo-derived plants after transplanting to Metromix in the greenhouse was increased when conversion of embryos had occurred on 5–20 μ*M* ABA. One-way analysis of variance showed $P = 0.002$. Means shown with different *letters* are significantly different at $P \leq 0.05$ according to Dunn's multiple comparison.

An average of 131 cotyledonary-stage embryos were available from each filtration using callus that had been maintained on either MS DCK or SH 2,4-D medium for culture on MS conversion medium. A *t*-test (unequal variance) was performed for comparing the means of maturation and conversion following maintenance of the callus on two different media (Table 1).

Filtered callus was cultured on either of the three maturation media (MS + AC, MS − AC, SH + AC), and there were 103–294 cotyledonary-stage embryos available from each filtration for culture on conversion medium. An analysis of variance followed by Dunn's multiple comparison with a 95% confidence interval ($P \leq 0.05$) was used for comparing the means of the treatments (SigmaStat, SPSS Inc., Chicago, IL) on embryo maturation and conversion (Table 2).

The ABA experiment showing the percentage of plants that survived consisted of three replicates in the greenhouse, and each replicate consisted of at least 10 plants that had been grown on each ABA concentration. An analysis of variance followed by Dunn's multiple comparison was used for comparing the means of the ABA treatments (Fig. 1). Tukey's multiple comparison was used for comparing the means of the ABA treatments on leaf size and plant DW (Fig. 3).

RESULTS AND DISCUSSION

Maintenance of embryogenic callus. Almost three times as many embryos matured from callus of 'Kardinal' maintained on the SH

FIG. 2. 'Kardinal' plants grown on conversion medium containing ABA grew more vigorously in culture and in the greenhouse. *Top*, Plants growing in Magenta jars on MS conversion medium without ABA (two plants on *right*) or with 20 μ*M* ABA (two plants on *left*). *Bottom*, Plants growing in the greenhouse after either growing on MS conversion medium without ABA (two plants on *left*) or with 20 μ*M* ABA (two plants on *right*).

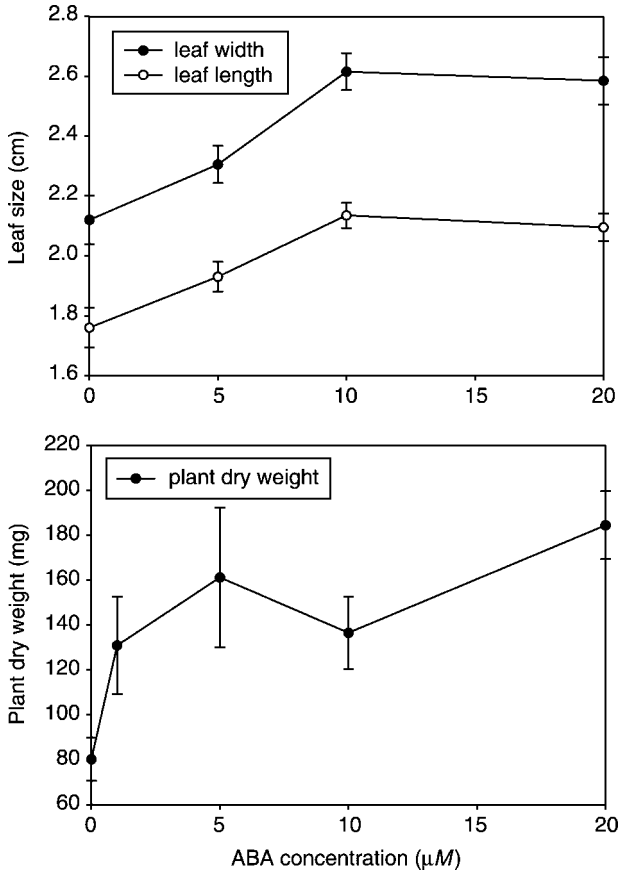

FIG. 3. Plants regenerated from cotyledonary-stage embryos grown for 2 mo. on MS conversion medium containing 5–20 μ*M* ABA had larger leaves and a higher dry weight than plants grown on the same medium without ABA. One-way analysis of variance showed $P < 0.001$ for leaf width and length and $P = 0.010$ for dry weight.

2,4-D medium as compared to callus maintained on MS DCK medium (Table 1). In comparison, cotyledonary-stage embryos from the callus maintained on MS DCK medium converted at a significantly higher rate (10 times) than embryos from the callus maintained on SH 2,4-D medium (Table 1).

Embryo maturation and conversion. AC in the MS maturation medium was detrimental to maturation of the embryos, possibly because an auxin was necessary for development of the embryo to the cotyledonary stage (Table 2). Conversion of cotyledonary-stage embryos to form plants was enhanced five times when the embryos had matured on MS, rather than SH, maturation medium containing AC (Table 2). AC in the MS maturation medium had no effect on conversion (Table 2).

Effects of ABA. ABA (10 or 20 μ*M*) in the MS maturation medium decreased the number of embryos that matured from filtered callus, and ABA (5–20 μ*M*) had no effect on the percentage of embryos that converted to form plants (data not shown). In comparison, ABA added to MS conversion medium significantly enhanced the number of plants that survived following transplantation to soil in the greenhouse (Figs. 1 and 2). The ability to survive may have been due to the increased DW of plants grown on 5–20 μ*M* ABA (Fig. 3). The increase in weight may be attributed to the larger leaf size, taller height, and more vigorous root growth for plants grown on 5–20 μ*M* ABA as compared to plants grown on conversion medium lacking ABA. Plants grown on 5–20 μ*M* ABA averaged 160 mg DW per plant as compared to 80 mg for plants grown without ABA (Fig. 3). ABA is known as a plant growth inhibitor but has recently been reported to promote growth in the absence of severe stress (Cheng et al., 2002). There was a significant difference in the size of leaves for plants grown for 6 wk on 10 and 20 μ*M* ABA as compared to plants grown with either 5 μ*M* ABA or without ABA (Fig. 3). The number of leaves per plant was the same (11.0) for plants grown on either 0 or 20 μ*M* ABA (data not shown). Plants grown on 5–20 μ*M* ABA were more vigorous with a better developed root system than plants grown without ABA. After 2 mo. of growth in the greenhouse, ABA-grown plants were typically taller (10.1 cm for ABA-grown plants as compared to 8.8 cm for the control plants not treated with ABA), and the flowers of ABA-grown plants developed faster (40–73% of the ABA-grown plants had flowers or flower buds as compared to 25% for control plants) although these trends were not statistically significant.

In conclusion, it is recommended that embryogenic callus of 'Kardinal' be maintained on MS DCK. Maximum maturation of embryos to the cotyledonary-stage occurred when callus was cultured on MS maturation medium lacking hormones. ABA (5–20 μ*M*) included in the MS conversion medium resulted in a higher (two times) survival rate when plants were transplanted to soil in the greenhouse. A conversion rate for 'Kardinal' of 15–30% was attained, and 90% of the regenerated plants survived in the greenhouse.

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